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Structural Characterization of the Novel and Thermal Stable Hydrogenases from the Purple Sulfur Bacteria *Thiocapsa roseopersicina and Lamprobacter modestohalophilus*

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14. ABSTRACT

This report results from a contract tasking Institute of Basic Biological Problems of RAS as follows: The hydrogenase is a key enzyme of the hydrogen metabolism of different microorganisms, catalyzing the reversible activation of molecular hydrogen. The use of this enzyme in biotechnology is often limited by its low thermostability and sensitivity to oxygen and other toxic agents. We have found that phototrophic sulfur bacteria possess very stable hydrogenases which can be used for the development of fuel cells and various photocatalytic systems for H2 production.

The overall goal of the project was the biochemical and structural characterization of hydrogenases isolated and purified from the cells of the noted above phototrophic bacteria. The specific aims included: (i) the cultivation of T. roseopersicina and L. modesohalophilus in photobioreactors under optimal conditions for high output of biomass; (ii) the development of effective methods for hydrogenase purification and biochemical and catalytic characterization; (iii) obtaining the hydrogenase crystals and study of their structure.

The main results obtained in the course of the Project where: 1) a large-scale cultivation of *T. roseopersicina, L. modestohalophilus* and *Ch. aurantiacus* and isolation of hydrogenase from these phototrophic bacteria allowing to obtain homogeneous preparations of the enzyme with a yield of 40-50% of the activity are developed and optimized; 2) it was discovered by the method of cryo-electron microscopy that a molecules of hydrogenase from *T. roseopersicina* form a ring-shaped hexameric complex with D3 symmetry. It is assumed that the formation of such hexameric structures provides increased stability of the enzyme to the action of temperature and proteases; 3) it is shown that hydrogenase *T. roseopersicina* exhibits high stability in the buffer systems, in which its molecules have a negative charge: at the pH range 7-9 and low ionic strength; 4) conditions for the crystallization of hydrogenase from *T. roseopersicina* providing production of the crystals of size to 0.2 mm on edge and diffraction of 3.2 Å are selected.

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Structural characterization of the novel and thermal stable hydrogenases from the purple sulfur bacteria *Thiocapsa roseopersicina* and *Lamprobacter modestohalophilus*

Final Project Technical Report

on the work performed from September 1, 2007 to May 31, 2011

Institute of Basic Biological Problems of RAS
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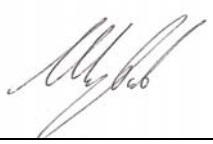
Director	Shuvalov Vladimir Anatolievich Academician	

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Keywords: phototrophic bacteria, *Thiocapsa roseopersicina, Lamprobacter modestohalophilus*, isolation and purification of enzymes, hydrogenase, hydrogenase crystals, spatial structure, thermal stability.

LIST OF CONTENTS

1. Brief description of the work plan: objective, expected results, technical approach

The aim of the project: development of methods of obtaining highly purified preparations of *Thiocapsa roseopersicina* and *Lamprobacter modestohalophilus* hydrogenases and structural characterization of these enzymes.

Expected results, technical approach

Task 1. To develop the bioreactor and to cultivate purple sulfur bacteria *T. roseopersicina* and *L. modestohalophilus*

Stage 1. Development of an appropriate photobioreactor for cultivation of purple sulfur bacteria *T. roseopersicina* and *L. modestohalophilus*

Stage 2. Selection of conditions for the operation of photobioreactor with a maximum yield of bacterial biomass

Task 2. To develop large-scale extraction and purification of hydrogenases from *T. roseopersicina* and *L. modestohalophilus*

Stage 1. Optimization of the extraction and purification of hydrogenase from *T. roseopersicina*

Stage 2. Development of the method of extraction and purification of hydrogenase from *L. modestohalophilus*

Stage 3. Development of the method for the extraction and purification of hydrogenase from the thermophilic green bacteria *Chloroflexus aurantiacus* under reducing conditions.

Task 3. To obtain crystals and to study the 3D structure of hydrogenases from *T. roseopersicina*

Stage 1. Optimization of conditions for crystallization of the hydrogenase

Stage 2. Investigation of the mechanism of hydrogenase inactivation at elevated temperatures

Stage 3. Study of the 3D structure of the hydrogenase

2. Method, Experiments, Theory etc.

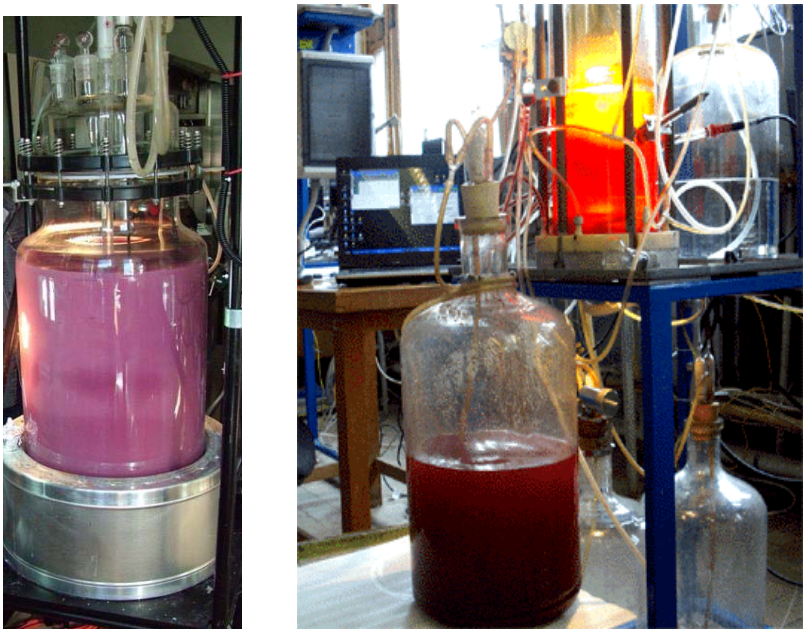
Methods of cultivation of *T. roseopersicina* and *L. modestohalophilus*

To perform large-scale production of biomass of *T. roseopersicina* and *L. modestohalophilus* we used two approaches: the first - deep cultivation in 100 L bioreactor with external lighting and the second - the cultivation of a thin layer photobioreactor based on coaxial glass cylinders.

A modified 100 L bioreactor company ACE GLASS was used for deep cultivation. Sterilization of the total volume of liquid in the flask 100 L bioreactor was carried out by heating with an external heating jacket. During the cooling fluid volume was blown with an inert gas (argon or nitrogen) aseptically through a membrane filter and specially mounted glass fitting to maintain the sterility of the medium and the anaerobic conditions of cultivation. Mixing of the total culture medium was performed using the built-in stirrer mixing of different speed to prevent the formation of the bacterial film on the walls of the flask of bioreactor. Additional components of the medium and inoculum were added into a stream of inert gas to prevent contamination of the medium. During the growth of bacteria an inert gas purge was carried out for 10-15 minutes every 2 days to maintain anaerobic conditions. Bioreactor was illuminated by portable fluorescent lamps installed on different levels around the flask of bioreactor (Fig. 1A)

For creation of a model of a thin layer photobioreactor three coaxial glass cylinders in height of 1 m closed by common bottom and common top lids have been made. The space between cylinders 1 and 2 was the outer chamber for cultivating phototrophic bacteria with working volume approximately 7 liters and thickness of the layer about 12 mm. Before adding the growth medium and inoculation, this space was sterilized with hot steam. The internal cylindrical layer

between the internal and middle cylinders was used as a temperature-regulating water-jacket. To illuminate the cultures of microorganisms a garland of several lamps is applied in inner cylinder. Mixing the cultures is implemented by sparging argon through the bottom lid of photobioreactor. Built - in outer camber pH and temperatures sensors allowed controlling these parameters during growth of bacteria. Addition of the sterile growth medium and inoculation was carried out through the bottom lid using peristaltic pump. Maintenance of constant volume of culture in photobioreactor was achieved with standard device - a discharge on a liquid level in sterile bottle for yield of the biomass located below a level of bacterial suspension in outer chamber. On the basis of the model of photobioreactor for cultivation of phototrophic microorganisms in the thin layer the pilot installation has been created (Fig. 1B).



A B

Fig 1. Photobioreactors for deep (A) and continuous (B) cultivation of phototrophic bacteria

Method of large-scale extraction and purification of hydrogenases from *T. roseopersicina*

The method for purification of *T. roseopersicina* hydrogenase worked out previously was used for small-scale biomass processing (not more then 100 g). This is due to the fact that the amount of biomass was limited by a capacity of chromatographic columns with protein contents in fractions. Since *T. roseopersicina* hydrogenase possesses heightened thermostability in comparison with most part of soluble proteins in cell fractions the stage of heating of cell-free extract was included into purification protocol. This allows to remove thermolabile proteins and to simplify the hydrogenase purification. Optimal regime of heating was chosen: 8-10 min of gradual rise in temperature of the extract up to 75^{o}C and 5 min of keeping of the extract at this temperature with subsequent fast cooling up to room temperature. This procedure provided removal of more than 60 % proteins from the extract at preservation of about 90 % of hydrogenase activity. Use of this stage allowed increasing the amount of initial biomass, i.e. scale of isolation, approximately three times.

Maximal homogeneity of the hydrogenase is main requirement for successful crystallization of the enzyme. Combination of different methods of a liquid chromatography on various carriers (ДЕАЭ-cellulose, Phenyl - Sepharose, Sephacryl, etc.) did not allow producing the hydrogenase preparation of a high degree of purity. The necessary degree of purification was achieved using an analytical electrophoresis in polyacrylamide gel (PAGE) only as a final stage. We have developed the preparative type of PAGE, which allowed producing up to 20 mg of a homogeneous hydrogenase in the final stage. For this purpose a glass column which has internal space for gel polymerization and two shirts (external and internal) for circulation

of a cooling liquid was used. Area of gel in a column make ~10 sm^2, it allows to apply simultaneously up to 5 ml of hydrogenase preparation. The hydrogenase obtained on this stage had a specific activity more than 100 U/ml and a high degree of homogeneity as control analytical SDS PAGE evidenced.

Method for extraction and putification of hydrogenase from *L. modestohalophilus*

Cells grown in the photobioreactor (100 g) were suspended in 100 ml of distilled water and treated with cooled acetone (1.2 l) for 30 min. The pellet obtained by filtration through a porous glass filter was washed with cooled acetone and dried to a powder. The acetone powder was suspended in 400 ml of distilled water and exposed by ultrasonic treatment (3 x 7 min). The first extract (I) was obtained by centrifugation of the suspension. The second extraction (II) of hydrogenase was carried out by the same way using the pellet after centrifugation, which was suspended in 150-200 ml of water. Both extracts were combined and saturated by ammonium sulfate up to 20 % of saturation. After centrifugation supernatant was heated on a water bath at 75^{o}C during 5 min. After centrifugation supernatant preparation obtained was subjected to a hydrophobic chromatography on Phenyl-Sepharose CL-4B (Pharmacia) column. Elution of hydrogenase from a column was carried out with 4 mM phosphate buffer, pH 7.0. Hydrogenase-containing fraction was chromatographed on ion-exchange column with Fractogel DEAE 650 (S) (Merck) and the enzyme was eluted from the column by linear gradient of NaCl (0 – 0.8 M). Fractions with hydrogenase activity were concentrated up to 2-ml using Centricon-30 (Amicon) and applied on a Sephacryl S-200 (Pharmacia) column. The proteins were eluted from the column with 25 mM phosphate buffer, pH 7.0 comprising 0.1 M NaCl. Fractions with hydrogenase activity were pooled and concentrated by ultrafiltration.

Methods of preparation of crystal and study of 3D structure of hydrogenase from *T. roseopersicina*

Previously, as a result of preliminary experiments on the structural characterization of stable hydrogenase from *T. roseopersicina* small crystals of cubic form of the enzyme (~ 0.04 mm on edge) have been obtained. They were formed in the presence of 0.1 M Tris buffer containing 1 M ammonium sulfate and 5% 2-methyl-2 ,4-pentanediol. Crystals of this size were not suitable for efficient X-ray analysis. In order to increase the size of the hydrogenase crystals more than 300 other systems of crystallization (Hampton Research Crystallization Screens, USA) were analyzed. A homogeneous preparation of hydrogenase with a concentration of 30 mg / ml was used in the experiments. Crystal growth was carried out in an incubator at 20 ° C using the methods of hanging and lying on the glass surface drop. The hydrogenase was crystallized in five different versions of the crystallization conditions. To study the spatial structure of hydrogenase the resulting crystals were mounted on the crystallographic loop and frozen in liquid nitrogen. Mounted crystals were subjected to X-ray radiation of high-intensity in Synchrotron Radiation Laboratory at the University of Stanford (SSRL). X-ray reflections obtained from the crystals were processed with the program HKL2000.

Supermolecular complexes of hydrogenase formed in solution were investigated by the method cryo electron microscopy and object reconstruction. For this hydrogenase preparations were dehydrated, frozen and examined under the electron microscope. The resulting micrographs were processed by a computer program the SPIDER to map the electron density of the molecule.

3. Results

3.1. Selection of conditions for the operation of photobioreactor with a maximum yield of bacterial biomass

At a deep cultivation *T. roseopersicina* was grown on Pfennig's medium at 30^{o}C, pH 7,4-7,6 and illumination I = 75 W m^{-2} in batch mode. The yield of wet biomass was about 3 g per 1 l.

To optimize growth parameters in thin-layer photobioreactor the experiments on continuous cultivation of *L. modestohalophilus* in turbidostat mode were carried out . Bacterium was

cultivated on Pfennig's medium at 30°C, pH 7,4-7,6 and illumination I = 50 W m^{-2}. To maintain anaerobiosis the culture in photobioreactor was sparged with argon (20 ml/min). pH was kept constant by addition of CO_2 in argon flow or NaOH solution by peristaltic pump. The biomass was collected in sterile bottle located below a level of the basis of photobioreactor. Cultivation monitoring and registration of growth parameters (pH, OD_{650}, t°C) were realized with the computer program (Iris).The cultivation of *L. modestohalophilus* in a turbidostat mode at different dilution rates was carried out and growth parameters of the culture and its hydrogenase activity were evaluated (Table 1). Results of experiments evidence that maximum biomass accumulation was observed at dilution rate of 0,06 h^{-1}, the yield of wet biomass was about 6 g/l. Specific hydrogenase activity of cells at all dilution rates remained approximately at the same level.

Table 1. Growth parameters and hydrogenase activity of *L. modestohalophilus* at different dilution rates.

Dilution rate, h^{-1}	OD_{650}	Bchl, μg/ml	Sulfur, μg/ml	Hydrogenase activity, nmol H_2/μg BChl
0.02	0.45	10.0	4.9	1.57
0.04	0.46	12.0	5.2	1.72
0.06	0.47	15.6	11.7	1.75
0.07	0.47	8.4	22.6	1.65

Sodium thiosulfate and sodium sulfide are main electron donors in metabolism of purple sulfur bacteria. To grow these microorganisms the modified Pfennig medium is commonly used, which contained large amount of sodium sulfide (1-2 g/l). It is metabolized in bacterial cells with S^o formation. High concentration of sulfide inhibits hydrogenase activity and surplus of sulfur complicates its purification. In order to determine optimal concentration of sulfide in growth medium we examined the influence of different concentrations of Na_2S on the growth and hydrogenase activity of *T. roseopersicina и L. modestohalophilus* (Table 2). The results obtained evidenced that the growth of both bacteria does not depend on sulfide concentration up to 1.0 g/l and is slowed down at its greater content. The hydrogenase activity of cells developed more intensively in the cultures growing at the sulfide concentration of 0.2 – 0.5 g/l, and was notably reduced already at 0.8 g/l. In conclusion, it is expedient to cultivate *T. roseopersicina* and *L. modestohalophilus* on the growth medium with the sulfide concentration not more than 0,5g/l.

Table 2. Effect of Na_2S in culture medium on the growth and hydrogenase activity of *T. roseopersicina* and *L. modestohalophilus.*

Na_2S, x 9 H_2O, g/l	***T. roseopersicina***		*L. modestohalophilus*	
	Bhl, g/l	Activity*, nmol MV/(min ml)	Bhl, g/l	Activity*, nmol MV/(min ml)
0	11,7	36,6	18,3	23,7
0,2	11,2	51,6	18,5	29,1
0,4	10,2	43,3	18,7	33,8
0,5	n.d.	n.d.	18,9	41,0
0,8	10,6	26,7	18,9	35,7
1,0	n.d.	n.d.	18,6	28,3

3.2. Optimization of extraction and purification of hydrogenase from *T. roseopersicina*

The developed method allowed to obtain 10 mg of homogeneous hydrogenase with a specific activity of 150 micromol / min * mg from 150 g wet biomass. Enzyme yield was approximately 44%. However, the use of hydrogenase purified according to this method did not provide a growth of crystals of a size sufficient for successful crystallographic studies. One possible reason for this could be a partial destruction of the distal cluster of hydrogenase during preparative electrophoresis in polyacrylamide gel. The appearance of iron in the frontal zone of the gel during electrophoresis may indicate on such destruction. Despite the fact that the protein part of the hydrogenase (appoprotein) was electrophoretically homogeneous, partially destroyed the structure of the enzyme might differ the native structure. For this reason, we have made some changes in an earlier elaborate scheme of purification: heating temperature of the enzyme preparations to remove thermolabile proteins was lowered from 75 ° to 70 ° C, and preparative electrophoresis has been replaced by the stage of gel filtration on a column of Sephacryl S-300 (Table. 3). This scheme of the purification allowed to increase the hydrogenase yield of 10% and to get larger crystals of the enzyme.

Table 3. Purification of thermal stable hydrogenase from *T. roseopersicina.*

Stage	Total protein, mg	Specific hydrogenase activity, μmol /(min * mg)	Total activity, μmol/min	Yield, %	Purification factor, fold
Crude extract	2700	1.12	3024	100	1
Fraction of 30-70% NH_2SO_4	1080	2.60	2756	91.0	2.3
Heating, 5 min, 75°C	412	5.40	2225	73.6	4.8
Hydrophobic chromatography on Phenyl-Sepharose CL-4B	81.4	24.2	1970	65.1	22
Ion-exchange chromatography on DEAE cellulose	28.2	65.8	1856	61.3	59
Gel-filtration on Sephacryl S-300	10.4	149.2	1552	51.3	133

3.3. Optimization of purification of the hydrogenase from *L. modestohalophilus*

Earlier we have presented the method of production of homogeneous hydrogenase from *L. modestohalophilus* cells with application of preparative electrophoresis in polyacrylamide gel (PAGE) at the last step. Unfortunately, this stage is quite hard and leads to partial loss in the enzyme activity. In order to improve the yield of pure hydrogenase the stage of PAGE was substituted for second ion-exchange chromatography on Fractogel DEAE 650(S) (Merck) column. The hydrogenase was eluted from the first column with Fractogel (2,0 x 20,0 cm) by wide linear gradient of NaCl – 0,0-0,8 M in 0,025 M phosphate buffer, pH 7,0. Fractions containing the hydrogenase were pooled, concentrated in Centricon-30 (Amicon) with dilution by the buffer for several times for removal of NaCl, and applied on the second Fractogel column (1,0 x 10,0 cm). This column with hydrogenase adsorbed was washed out by buffer

containing 0,2 M NaCl. Elution of the enzyme was carried out by linear gradient of sodium chloride - 0,2 - 0,6 M. The hydrogenase was washed off in a narrow zone in a range of NaCl concentration 0,4 - 0,5 M. Central fractions with hydrogenase activity did not practically contain any ballast proteins. The yield of hydrogenase on activity with use of this stage reached about 55%, that is 12-15% more then at PAGE application. The results of purification are shown in Table 4.

Table 4. Purification of thermal stable hydrogenase from *L. modestohalophilus*

Stage	Total protein, mg	Hydrogenase activity,		Yield, %	Purification factor, fold
		μmol H_2 /(min * mg protein)	μm/min		
1. Total extract (I +II)	640	0.71	455	100	1
2. Supernatant after $(NH_4)_2 SO_4$ precipitation (20%)	520	1.33	393	86	1.9
3. Supernatant after heating (75°C, 5 min).	210	1.53	310	68	2.2
4. Hydrophobic chromatography on Phenyl-Sepharose CL-4B	21.0	16.0	340	74	23
5. Ion-exchange chromatography on Fractogel DEAE 650(S) I	1.7	156.1	265	58	223
6. Ion-exchange chromatography on Fractogel DEAE 650(S) II	1.5	117	175	55	167

3.4. Optimization of the purification of hydrogenase complex from the thermophilic green bacteria *Chloroflexus aurantiacus*, strain the J-10-f1

In the period under project the development of extraction of Hox EFUYH hydrogenase from the cells of green filamentous bacteria *Ch. aurantiacus* has been undertaken. The hydrogenases of this type from other microorganisms are very sensitive to oxygen therefore all buffer solutions used were saturated with Ar and contained 2 mM dithiotreitol. The stages of purification are presented in Table 5. The most effective stages are two ion-exchange chromatographies on a column with Fractogel DEAE 650 (S). The central fractions of the hydrogenase eluted from the second Fractogel column practically did not contain some ballast proteins and were close to homogeneous preparations of the enzyme.

The hydrogenase preparations obtained using this purification method were able to interact as with artificial (methyl and benzyl viologens) and physiological (NAD, NADH) electron mediators (Table 6). This fact evidences that purified hydrogenase complex contains both hydrogenase (HoxYH) and diaphorase (HoxEFU) parts.

Stability of purified hydrogenase was evaluated as the activity of the enzyme preparations in reactions of acceptor-dependent H_2 uptake during storage under different conditions. The enzyme quickly loses activity, especially NAD-reducing, in the presence of oxygen. This allows concluding that the complicated native structure of hydrogenase can destroy due to oxidizing destruction of bounds between hydrogenase and diapphorase parts. Under anaerobic conditions both MV- and NAD-dependent hydrogenase activities decreased more slowly and by similar way.

Table 5. Purification of hydrogenase from the thermophilic green *bacteria Chloroflexus aurantiacus,* strain the J-10-f1.

Stage	Total protein, mg	Specific hydrogenase activity, μmol /(min * mg)	Total activity, μmol/min	Yield, %	Purification factor, fold
Crude extract	1200.0	0.25	297.6	100	1
Soluble fraction	620.0	0.43	265.4	89.0	2.0
Ion-exchange chromatography on Fractogel DEAE 650(S) I	44.0	3.8	168.5	56.6	15.2
Hydrophobic chromatography on Phenyl-Sepharose CL-4B	25.0	5.7	142.4	47.8	23
Ion-exchange chromatography on Fractogel DEAE 650(S) II	5.2	25.4	132.1	44.0	102

Table 6. Reactions catalyzed by purified hydrogenase *Ch. aurantiacus*.

Reaction*	The reaction rate (hydrogenase activity) μmol min^{-1} mg^{-1} protein**
H_2 evolution from MV^+ (2 mM)	20,8
H_2 evolution from NADH (3 mM)	0,22
Control (DT)	0,04
H_2 uptake in the presence of MV^{2+}(1 mM)	32,2
H2 uptake in the presence of BV^{2+} (1 mM)	38,0
H2 uptake in the presence of NAD(1 mM)	3,8

*Reaction systems contained 0.05M phosphate buffer, pH 7.0 (H_2 evolution) or 0.05 M Tris-HCl buffer, pH 8.5 (H_2 uptake), saturated with Ar, and 20 μl hydrogenase.
H_2 was injected in reactions as 100 μl buffer saturated with H_2.
** The reaction rates were calculated from kinetics measured with hydrogen electrode at 30°C.

3.5. Obtaining crystals and study the spatial structure of hydrogenase of *T. roseopersicina*

3.5.1. Effect of organic solvents on the stability of *T. roseopersicina* hydrogenase

Since the some mediums used for formation of hydrogenase crystals include organic solvents the factor of stability of the hydrogenase in the organic environment has critical value. To find out, what kind of organic solvents possess the least toxic action regarding hydrogenase, the enzyme purified from *T. roseopersicina* cells was incubated within day in the buffer solutions containing different concentration of some organic solvents, and its residual activity was examined in the standard test - H_2-dependent methyl viologen reduction. The results represent on Fig. 2 testify that this hydrogenase appeared rather stable in the presence of all solvents used at their concentration up to 40 %. In the cases of higher concentrations of alcohols (proton solvents) and DMSO (dimethyl solfoxid) residual activity of the enzyme was notably reduced. THF (tetrahydrofuran) showed inhibiting action on the enzyme in the range of 40-60 %, lower, as well as its higher contents in incubation medium had no influence. The hydrogenase

remained practically fully active in the mediums containing acetone or acetonitrile down to 99 %. Consequently, these solvents even in high concentration can be applied for the work with *T. roseopersicina* hydrogenase.

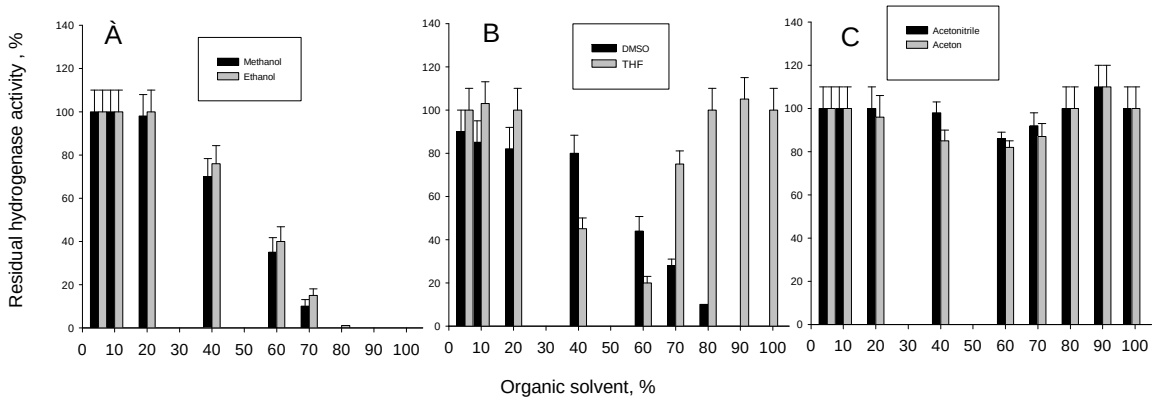


Fig. 2. Effect of preliminary incubation of *T. roseopersicina* hydrogenase at the presence of organic solvents on its activity in reaction of H_2-dependent MV reduction.

3.5.2. Study of the mechanism of inactivation of hydrogenase at elevated temperatures

Knowledge of the nature of high thermal stability of hydrogenases from purple sulfur bacteria is important both in theoretical and practical terms. Hydrogenase *T. roseopersicina* has high thermal stability: a relatively high rate of enzyme inactivation occurs at temperatures above 80 ° C. It is known that non-covalent interactions (salt bridges, hydrogen bonds, hydrophobic interactions, etc.) including surface electrostatic interactions cause the increased thermal stability of proteins. The change in pH and ionic strength buffers, as well as adding various stabilizing additives can have a significant effect on such interactions in hydrogenases. On the example of the hydrogenase from *T. roseopersicina*, we studied the effect of pH and ionic strength of buffer on kinetics of inactivation of the enzyme at 90 ° C. It should be noted that only under such conditions there is a relatively rapid inactivation of hydrogenase with $\tau_{1/2}$ of about 2 h. For example, at room temperature $\tau_{1/2}$ for this hydrogenase is more than six months and a month at 50 ° C, that considerably limits the possibility of studying the mechanism of thermal inactivation of the enzyme at these temperatures.

The results showed that when a molecule of hydrogenase has a negative charge (in Tris-HCl-buffer the pH range 7-9), the enzyme has high thermal stability. At pH closed to the isoelectric point of the enzyme (pI = 4.2), thermal stability was significantly reduced. So, at pH 5.0 the rate of thermal inactivation of hydrogenase was doubled compared to the control at pH 7.0 (Fig. 3 A). With increasing pH of the buffer to 9.0 thermal stability of the enzyme has remained virtually unchanged relative to controls. By increasing the ionic strength of buffer solution, positively charged ions can partially neutralize the negative charge on the surface of hydrogenase. Indeed, the addition of potassium chloride to the enzyme solution resulted in significant reduction of thermal stability of the enzyme (Fig.3 B). Thus, these results indicate the important contribution of the surface charge on the thermal stability of hydrogenase. A significant stabilizing effect on hydrogenase rendered hydrophobic additives, such as glycerol. We can therefore assume that hydrophobic interactions also make an important contribution to stabilizing the molecular structure of hydrogenase.

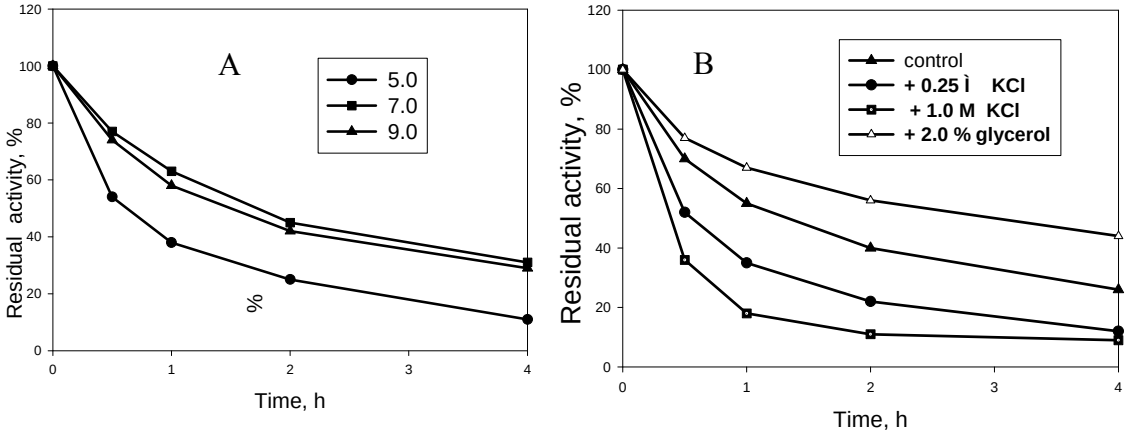
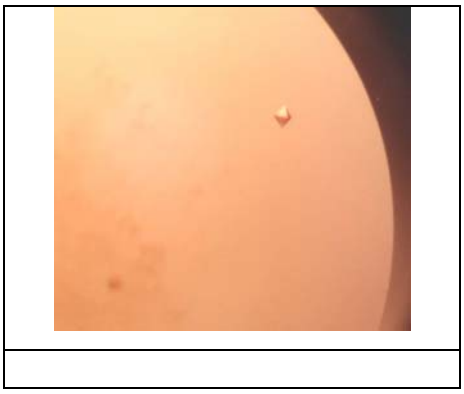


Fig. 3. Effect of pH (A), salt and glycerol (B) on the inactivation of *T. roseopersicina* hydrogenase at 90°C.

The inactivation kinetics of hydrogenase from the purple sulfur bacterium *T. roseopersicina* at raised temperatures was studied. Inactivation of the hydrogenase at 80°C under anaerobic conditions occurs in one step according to the first-order reaction with $k_1^i = 7x10^{-5}$ c^{-1}. Under aerobic conditions this process occurs in two steps, with a decrease in the rate at the late step ($k_2^i = 1.8 \times 10^{-6}$ c^{-1}). The immobilization of hydrogenase on different matrix did not result in increase of the stability of this enzyme at raised temperatures. The thyol reagent decrease the thermal stability of the hydrogenase and the enzyme denaturation was irreversible under these conditions. The hydrogenase partially inactivated under aerobic conditions can be reactivated after addition of the reducer – sodium dithionite. The data obtained indicate the important role of disulfide bonds in stabilization of native structure of hydrogenase from *T. roseopersicina*.

3.5.3. Optimization of crystallization conditions of hydrogenase from *T. roseopersicina* using different media and different additives

The study of more than 300 buffer systems for the crystallization of the hydrogenase from *T. roseopersicina* and optimization of their compositions found it possible to determine the composition of the optimal buffer system for growth of crystal with largest size and good quality of diffraction. Figure 4 shows a microphotograph of crystals obtained from a solution containing 0,01 M magnesium sulfate, 2.0 M ammonium sulfate and 0.05 M sodium kakodilate pH 6.5 and the solution of the hydrogenase with protein concentration of 10 mg / ml. Time of crystal growth in this medium was reduced up to 5-7 days and grew crystals of hydrogenase, obtained by diffusion-evaporation at 24 ° C, reach a size of 0.2 mm on edge.



Fifure 4. Crystal of hydrogenase from *T. roseopersicina*

3.5.4. A study of the spatial structure of hydrogenase *T. roseopersicina*

A study on the structure of hydrogenase in solution was performed by transmission electron microscopy techniques. On the micrographs a ring-shaped supermolecular structures of hydrogenase of 12 nm diameter can be seen. This size of complex corresponds to its hexameric structure. To further examine the supermolecular complex the hydrogenase was investigated by the methods of cryo-electron microscopy and object reconstruction. For this hydrogenase preparations were dehydrated, frozen and examined under the electron microscope. The resulting micrographs were processed by a computer program the SPIDER to map the electron density of the molecule (Fig. 5).

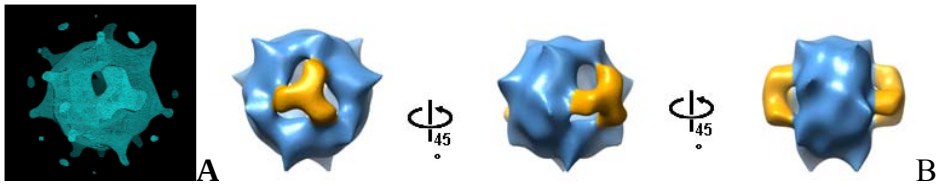


Fig. 5. Initial (A) and processed (B) maps of electron density of the hexameric structure of hydrogenase from *T. roseopersicina*.

It was found that the formation of supermolecular structures depends on the protein concentration in the hydrogenase. The formation of hexameric complexes was observed at high protein concentrations above 2 mg / ml. Dilution of hydrogenase preparations below 2 mg / ml resulted in a breakdown of hexameric and the formation of tetrameric, dimeric or trimeric oligomers. It was a challenge for processing cryo-electron photos, because at high concentrations of the enzyme the hydrogenase complexes were in different positions on the cryo grids. To resolve this problem, the dilution of concentrated hydrogenase samples was performed on the grids with quick dehydration and freezing.

Cryo-electon microscopy images of *T. roseopersicina* hydrogenase were obtained with the low passed fillers at 7Å and 15 Å which were used to create the electron density map. As a result of image reconstruction the electronic density was visible both in the ring and its central part. The electron density map showed that the supermolecular complex of hydrogenase is composed of 6 heterodimers, combined in hexameric structure with D3-symmetry, ie, 3 molecules of hydrogenase heterodimer located in one ring and three others - are located in another ring (Fig 5). In addition, 53 amino acids of C-terminus of the small subunit of hydrogenase which form a cap on both sides of hexameric assembly involved in maintaining its structure. Apparently, such a complex organization of the supermolecular structure of hydrogenase is responsible for the stability of the enzyme to high temperatures. It is shown that the C-terminal sequence of monomers in amidase from *Desulfovibrio gigas*, consisting of 81 amino acids involved in the formation of dimers, assembled in hexameric supermolecular structure. This organization of the protein molecule significantly increases the thermal stability of amidase from *D. gigas* in relation to other enzymes of this class (Fig. 6).

Fig. 6. Comparison of the amino acid sequences of small subunits of hydrogenases from *T. roseopersicina* and *D. gigas* (A) and cryo-map (B). Red square marked with a sequence of the C-terminus, which may be involved in the formation of the cap.

The crystals of hydrogenase from *T. roseopersicina* have been obtained in an optimized solution consisting of 0,01 M magnesium sulfate, 2.0 M ammonium sulfate and 0.05 M sodium kakodilate pH 6.5 and a hydrogenase solution with protein concentration of 10 mg / ml (Fig. 5). For their study the crystals were mounted on the crystallographic loop and frozen in liquid nitrogen (Fig. 7).

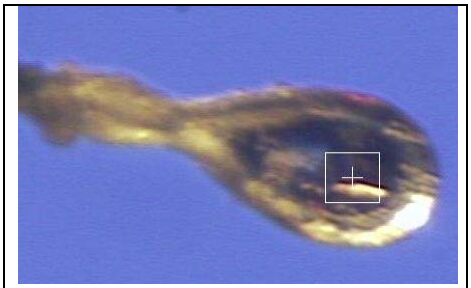


Fig. 7. The crystals of hydrogenase *T. roseopersicina* in the cryogenic loop. White square indicates the size of the X-ray

Mounted crystals were subjected to X-ray radiation of high-intensity at Synchrotron Radiation Laboratory of the University of Stanford (SSRL) on the line BL 9-1 and 9-2. X-ray reflections derived from crystals were processed with a software package HKL2000. Data collection results have shown that we managed to obtain the resolution of crystals up to 3,2 Å (Table 7). Crystals have rhombohedral crystalline lattice with hexagonal parameters of the elementary cell and may be belong to spatial group of symmetry R32:H. The elementary cell of crystals has the following parameters a=120 Å, b=120 Å, c=295 Å, α =90, β =90, γ =120.

Mathematical processing of reflection data by function of autorotation indicates the presence of two crystallographic axes in the fold *Chi* 180 ° (Fig. 8 A) and three non-crystallographic axes of the bend at *Chi* 120 ° (Fig. 8 B).

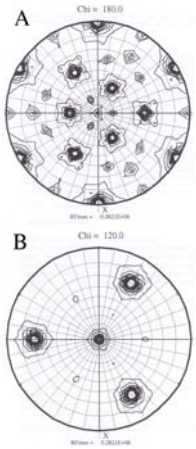


Fig 8. Self rotation plots corresponding (A) *Chi* 180° and (B) *Chi* 120°

These data are consistent with the presence of three heterodimers, reflecting the D3 symmetry (hexamer) in the monoclinic space unit cell.

In order to obtain phase angles of reflection of electrons we used the method of molecular replacement (program package SSR4). In this case the phase are determined by applying a known protein structure to the experimentally derived electron density. However, this method did not give positive results that may be stipulated by a higher percentage of probability of non-compliance of the search model, or, more likely, by a chaotic arrangement of the molecules in the desired structure in the crystal.

Therefore, we have used other search methods. Such methods are the method of combining the Multiple anomalous diffraction (MAD) and single anomalous diffraction (SAD). For the MAD experiments the iron located in the iron-sulfur clusters of hydrogenase studied was used. However, the low resolution of the crystals and the poor signal strength of the iron atoms did not let to determine the exact location of the iron atoms and the phase angles. Recently the data on the use of cluster of tantalum bromide, having a high strength of the anomalous reflection at a sufficiently low diffraction of crystals were published. At a resolution of 5,5-7,0 Å tantalum cluster reflects the electrons as a single super atom. We decided to use this approach for studying the structure of the hydrogenase from *T. roseopersicina*. To do this, the crystals were saturated with 1mM solution of tantalum bromide and washed mounted on a cryo loops and frozen in liquid N_2. Summary of data collection at the Br peak edge (0.919 Å) and processing are presented in Table 8.

Table 7. Data collection statistics for crystals of hydrogenase from *T. roseopersicina*.

Sample	Native data
Wavelength Used (Å)	0.8855
Space Group	R32
Resolution (Å)	50-3.95
Total Number of Reflections	64646
Number of Unique Reflections	7593
Rsym (I)	0.078
Rsym in Highest Res. Shell	0.218
Completeness (%)	100

Using different software (software packages SSR4, Phinex, Solve / Resolve), we obtained the coordinates of the clusters of tantalum in the crystals of hydrogenase. Combining the methods of molecular replacement, SAD and MAD allowed us to get the first electron density map and the initial coordinates of the molecules of hydrogenase from *T. roseopersicina*. These results are consistent with earlier data on cryo reconstruction hexameric hydrogenase complexes (Fig. 9).

Table 8. Data collection statistics for crystals of hydrogenase from *T. roseopersicina*

Sample*	Native	Fe edge		$Ta_6Br^{2+}_{12}$ (Br edge)
		Peak	Inflection	
Wavelength Used (Å)	0.8855	1.7381	1.7413	0.9194
Space Group	R32	R32	R32	R32
Resolution (Å)	50-3.6	50.0-5.3	50.0-4.7	100-5,9
Total Number of Reflections	64646	30210	47793	10720
Number of Unique Reflections	7593	3224	4607	2435
Rsym (I)	0.078	0.113	0.135	0.038
Rsym in Highest Res. Shell	0.218	0.410	0.487	0.315
Completeness (%)	100.0	99.7	99.5	99.7

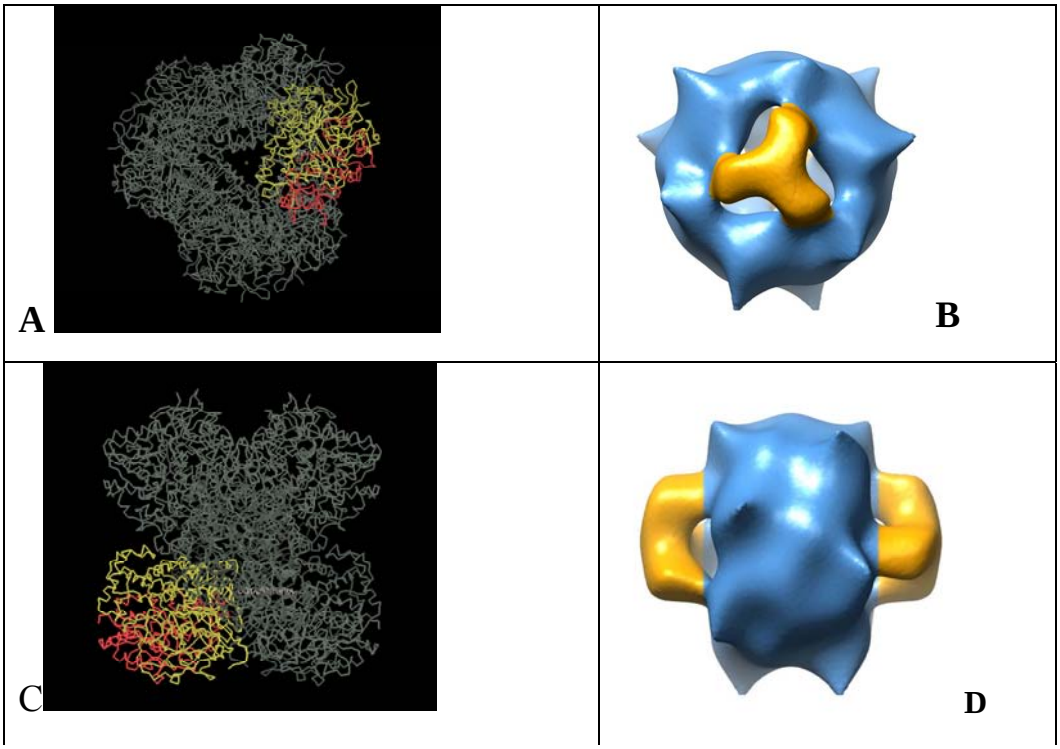


Fig. 9. Structural characterization of hydrogenase from *T. roseopersicina*. A, C - initial model, obtained after combining MAD and SAD methods. B, D - a model of the enzyme after the reconstruction by methods of cryo-electron microscopy. A and B are 3-fold symmetry, and C and D - 2-fold symmetry of hexameric structure.

Thus, during the time under Project the crystals and the optimal conditions of crystallization of hydrogenase were obtained. For the first time the initial phases of the reflection angles and coordinates of the model structure of the hydrogenase from *T. roseopersicina* were determined using the methods of X-ray structural analysis.

4. Conclusion

The main results obtained in the course of the Project:

- A large-scale cultivation of *T. roseopersicina, L. modestohalophilus* and *Ch. aurantiacus* and isolation of hydrogenase from these phototrophic bacteria allowing to obtain homogeneous preparations of the enzyme with a yield of 40-50% of the activity are developed and optimized.
- It was discovered by the method of cryo-electron microscopy that a molecules of hydrogenase from *T. roseopersicina* form a ring-shaped hexameric complex with D3 symmetry. It is assumed that the formation of such hexameric structures provides increased stability of the enzyme to the action of temperature and proteases.
- It is shown that hydrogenase *T. roseopersicina* exhibits high stability in the buffer systems, in which its molecules have a negative charge: at the pH range 7-9 and low ionic strength.
- Conditions for the crystallization of hydrogenase from *T. roseopersicina* providing production of the crystals of size to 0.2 mm on edge and diffraction of 3.2 Å are selected.

5. References

Attachment 1: List of published papers and reports with abstracts

1.Serebryakova L.T., Zorin N.A., Karyakin A.A. Improvement of hydrogenase enzyme activity by water-miscible organic solvents. *Enzyme and Microbial Technology*, **44**, 329 (2009).

Abstract

Both stability and catalytic activity of the HynSL *Thiocapsa roseopersicina* hydrogenase in the presence of different water-miscible organic solvents were investigated. For all organic solvents under study the substantial raise in hydrogenase catalytic activity was observed. The stimulating effect of acetone and acetonitrile on the reaction rate rose with the increase in solvent concentration up to 80 %. At certain concentrations of acetonitrile and acetone (60-80% v/v in buffer solution) the enzyme activity was improved even 4-5 times compared to pure aqueous buffer. Other solvents (aliphatic alcohols, dimethylsulfoxide and tetrahydrofuran) improved the enzyme activity at low concentrations and caused enzyme inactivation at intermediate concentrations. The long-term incubation of the hydrogenase with aliphatic alcohols, dimethylsulfoxide and tetrahydrofuran at intermediate concentrations of the latter caused enzyme inactivation. The reduced form of hydrogenase was found to be much more sensitive to action of these organic solvents than the enzyme being in oxidized state. The hydrogenase is rather stable at high concentrations acetone and acetonitrile during long-term storage: its residual activity after incubation in these solvents upon air within 30 days was about 50 %, and immobilized enzyme remained at the 100% of its activity during this period.

2. Zadvorny O.A., Barrows A.M., Zorin N.A., Peters J.W., Elgren T.E. High level of hydrogen production activity achieved for hydrogenase encapsulated in sol–gel material doped with carbon nanotubes. *J. Mater. Chem.,* **20,** 1065 (2010).

Abstract

Doping hydrogenase-containing sol-gel materials with multi-walled carbon nanotubes, polyethylene glycol and methyl viologen results in a greater than 50% increase in hydrogen production achieving levels of hydrogenase activity comparable to that observed optimally in aqueous solution.

3. Zadvorny O.A., Allen M., Brumfield S.K., Varpness Z., Boyd E.S., Zorin N.A., Serebriakova L., Douglas T., Peters J.W. Hydrogen Enhances Nickel Tolerance in the Purple Sulfur Bacterium Thiocapsa roseopersicina. Environ. Sci. Technol. 44, 834, (2010).

Abstract

A common microbial strategy for detoxifying metals involves redox transformation which often results in metal precipitation and/or immobilization. In the present study, the influence of ionic nickel [Ni(II)] on growth of the purple sulfur bacterium, Thiocapsa roseopersicina was investigated. The results suggest that Ni(II) in the bulk medium at micromolar concentrations results in growth inhibition; specifically, an increase in the lag phase of growth, a decrease in the specific growth rate, and a decrease in total protein concentration when compared to growth controls containing no added Ni(II). The inhibitory effects of Ni(II) on the growth of T. roseopersicina could be partially overcome by of the addition of hydrogen (H_2) gas. However, the inhibitory effects of Ni(II) on the growth of T. roseopersicina were not alleviated by H_2 in a strain containing deletions in all hydrogenase-encoding genes. Transmission electron micrographs of wild-type T. roseopersicina grown in the presence of Ni(II) and H_2 revealed a significantly greater number of dense nanoparticulates associated with the cells when compared to wild-type cells grown in the absence of H_2 and mutant strains lacking hydrogenase activity grown in the presence of H_2. X-ray diffraction and vibrating sample magnetometry of the dense nanoparticles indicated the presence of zero-valent Ni, suggesting Ni(II) reduction. Purified T. roseopersicina hyn-encoded hydrogenase catalyzed the formation of zero valent Ni particles in vitro, suggesting a role for this hydrogenase in Ni(II) reduction in vivo. Collectively, these results suggest a link between H_2 metabolism, Ni(II) tolerance, and Ni(II) reduction in T. roseopersicina.

4. Gogotov I.N., Zadvorny O.A., Zorin N.A., Serebryakova L.T. Bacterial hydrogenases. *In: Proceedings of Winogradsky Institute of Microbiology, issue 15: Photosynthetic microorganisms* (Ed. By Galchenko) – Moscow: MAKS Press., 2010, p. 260-289.

Abstract

Hydrogenases – enzymes catalysing the reversible oxidation of molecular hydrogen – play a central role in microbial energy metabolism. The basic characteristics of different type hydrogenases synthesized in chemotropic and phototrophic bacteria are under consideration in this review: classification, the reactions catalyzed, the functions performed in a metabolism of microorganisms etc. Depending on metal ions contained in their active sites, these enzymes fall into three main classes: [NiFe]-, [FeFe] and [Fe]- hydrogenases. [NiFe] – hydrogenases are represented in details as the most numerous class. On a basis of the physiological function the hydrogenases of this class are divided into four large groups. Each group in accordance with the homology of hydrogenase structure genes unites several subgroups. The modular model of the arrangement on hydrogenase molecule is supposed. It consists of three parts: strict conservative H_2- binding module; relatively conservative module which performs intramolecular electron transfer; and the varying module providing interaction with electron carriers. Present view of the active site structure of [NiFe]-hydrogenases and the mechanism of catalysis are reflected. The data on the structural and accessory genes responsible for synthesis of hydrogenases with different functions, and regulation of this process are summarized. The special attention is concentrated on the comparative analysis of stability of hydrogenases from phototrophic bacteria *in vitro* and their resistance to various denaturing factors. It is shown that the hydrogenases may participate in redox transformation of metals and anaerobic corrosion of metals. Biotechnological opportunities of application of hydrogenases are considered: systems for H_2-production, fuel cells, systems for remediation of sewage etc.

Attachment 2: List of presentations at conferences and meetings with abstracts

1. Zorin NA, Zadvorny OA, Serebryakova LT. Hydrogen-activating biocatalysts on the basis of hydrogenase from phototrophic microorganisms. Abstracts of Russian symposium "Autotrophic microorganisms" (23-26 December 2010) Moscow, MAKS Press.

Abstract

The formation of H_2 by materials produced with encapsulation of the homogeneous *T. roseopersicina* hydrogenase and of multi-walled carbon nanotubes in silica gel was studied. It is shown that in such material between the nanotubes and the active site of the enzyme the direct electron transfer has place. It is established that a combination of nanotubes and metilviologen in gel increases significantly the efficiency of the catalytic action of hydrogenase. A comparative analysis of the immobilization of homogeneous hydrogenase *T. roseopersicina* on the surface of single-walled and of multi-walled carbon nanotubes (CNTs), whose surfaces was modified by various active groups for more efficient binding of the hydrogenase was performed. It is established that the CNT-modified ditiopyridil grou have several advantages over other types of nanotubes. These CNTs form a stable monolayer which can be transferred to the conductive matrix using the Langmuir-Blodgett (LB) method. LB films on the electrode was used as a matrix for the immobilization of hydrogenase to form a nanocomplexes CNT-ditiopiridil-hydrogenase. The electrochemical properties of the composites were characterized.

2. Oleg Zadvornyy, Kevin Swanson, Timothy E. Elgren, Liang Tang, J, Nikolay Zorin, Trevor Douglas, and John W. Peters. Hybrid Systems for Photocatalytic Hydrogen Production. Gordon Research Conference “Iron-Sulfur Enzymes” (June 6-11, 2010) Colby-Sawyer College, New London, NH, USA.

Abstract

The exposed surfaces of the protein-based H_2 producing catalyst systems (both the hydrogenase and synthetic protein cage systems) provide a rich template for covalent attachment of light harvesting antennae systems including either molecular, colloidal, or solid surfaces. The overall goal is to maximize the light harvesting capacity of each self-assembled protein-based catalyst for optimized and directed electron transfer to the protein catalyst. For the hydrogenases covalent attachment is not simple since the ability to heterologously express the enzymes of interest in a manner that allows the purification of large amounts of homogenous preparations of enzyme is not straightforward. We have therefore been using a two pronged approach involving pilot work targeted at providing a preliminary analysis of the effects of coupling model light harvesting complexes to both [NiFe]- and [FeFe]-hydrogenases that are the targets of our studies and in parallel improving the means to express hydrogenase enzymes and engineer modification sites in an informed manner.

3. JW Peters, O Zadvornyy, SE McGlynn, SS Ruebush, DW Mulder, EM Boyd, and T. Beard 2008. Annual activities of Peters Subgroup of the BioSolar H_2 Team (August 5-6) Arlington, VA. AFORSR Biofuels Program Review.

Abstract

There is a tremendous need to expand our knowledge base of hydrogenase structures to better understand the fundamental basis behind various properties of these enzymes including oxygen sensitivity, intermolecular and intermolecular electron transfer, and propensity of certain hydrogenases to catalyze proton reduction versus hydrogen oxidation and vise versa. These enzymes are traditionally very hard to work with since many are sensitive to oxygen and cannot be heterologously expressed in a facile manner. Our group has specific expertise in the purification and characterization of hydrogenases and nitrogenases and has made a number of seminal contributions in both areas. The two priorities in the current project for structural

characterization are the 1) [FeFe]-hydrogenase from *Chlamydomonous reinhardtii* and 2) the structural characterization of [NiFe hydrogenases] from phototrophic organisms. We are focusing on identifying suitable [NiFe] targets from various phototrophs and determining their structures. Given the large diversity of properties of [NiFe]-hydrogenase structures and that the only structurally characterized examples are derived from sulfate reducing bacteria, an imperative has been placed on the structure determination of additional [NiFe]-hydrogenases.

4. Oleg Zadvorny, Liang Tang, Susan Brumfield, Joe Fox, Matt Prisse, Nikolay Zorin, Trevor Douglas and John Peters. Towards the Determination of the Structure of the Stable [NiFe] Hydrogenase from the Purple Sulfur Bacteria *Thiocapsa roseopersicina*. Hydrogenase and hydrogen production: The 8th International Hydrogenase Conference (August 5-10, 2007), Breckenridge, Colorado, USA. <http://www.chem.tamu.edu/hydrogenase/abstracts.pdf, P95 >.

Abstract

The NiFe hydrogenase from *Thiocapsa roseopersicina* is being studied in the lab for its potential in generating bioinspired materials for hydrogen production. The structural characterization of this enzyme will contribute to the understanding of the determinants for oxygen and thermal stability, and pave the way for engineering targeted amino acid substitutions. Transmission electron microscopy data and cryoelectron microscopy and image reconstruction experiments of *T. roseopersicina* hydrogenase indicates that it is organized into a supermolecular complex consisting of 2 agglomerated rings composed of three heterodimers. Small cubic crystals (~0.05 mm on an edge) have been obtained however the crystals diffract poorly (~5 Å resolution) and thus a structure solution has not yet been possible. Using low resolution diffraction data and self rotation function we found that the supermolecular complex of hydrogenase has 2, 3 and 6-fold non crystallographic symmetries, which corresponds with the cryo image reconstruction data and confirms the hexameric organization of the hydrogenase. Progress toward the structure of this unique hydrogenase will be presented in addition to recent results on the application of this stable hydrogenase in hydrogen producing materials.

Attachment 3: Information on patents and copy rights (List and describe patents and copyrights which were obtained or may be obtained as a result of the project)

No